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(54) Title: METALLO PROTEINASE INHIBITORY AGENT

(57) Abstract: The present invention relates to an active agent, in particular a metalloproteinase inhibitory agent comprising casein-derived peptides obtained by hydrolysis of casein by a food grade bacteria. The invention also relates to the manufacture of a agent, in which a food grade bacteria of the genus Lactobacillus helveticus is contacted with casein in order to perform an hydrolysis and obtain casein-derivated peptides exhibiting metalloproteinases inhibitory property. The present invention also concern the isolated and purified inhibitory peptides obtained by hydrolysis of casein by the said bacteria.

METALLO PROTEINASE INHIBITORY AGENT

The present invention relates to an active agent, in
particular a proteinase inhibitory agent. More
specifically, this invention relates to metalloproteinase
inhibitory agent comprising casein-derived peptides.

The degradation of extracellular matrix (ECM) is a very
complex process and is part of many pathological and
physiological processes. The proteolytic degradation of the
extracellular matrix therefore plays a crucial role in
cancer invasion as also in non-neoplastic tissue
remodelling processes. The role of the matrix-
metalloproteinases (MMPs) enzymes in the tumor cell-
mediated extracellular matrix proteolysis is well
established. Thus, by regulating composition and integrity
of ECM, MMPs play an important role in the control of
signals elicited by matrix molecules and regulate cell
proliferation, differentiation and cell death. MMPs are
ubiquitous enzymes and prostate, breath, bone as well as
colon tumor cell lines have been shown to secrete MMPs in
vitro. The MMP familly has over 20 members that cleave
various components as various as fibronectin, gelatin and
collagen for exemple. Among the MMPs familly, MMP-2 and
MMP-9 have been identified in cancer cells and the active
froms were detected more frequently in malignant than in
benign breast carcinoma. MMP-2 may thus be considered as a
key enzyme in ECM remodeling involved in tumor invasion and
metastasis.

Beyond cancer and tumor invasion MMPs are also associated
with a wide variety of normal and pathological conditions
involving matrix degradation and remodeling. MMPs are thus
involved in some physiological processes like foetal
development, angiogenesis, bone formation and resorption.
Indeed, bone resorption models showed that MMP-9 is
specifically required for the invasion of osteoclasts and
endothelial cells into the discontinuously mineralized
hypertrophic cartilage that fills the core of the
diaphysis. MMPs have also been reported to be involved in
pathological processes like artherosclerosis, arthritis,
autoimmune diseases, periodontitis, tissue ulceration and
of course cancer invasion and metastasis.

It is also known that the proteolytic activity of the MMPs
involved in ECM degradation may be regulated by their
endogeneous inhibitors, the tissue inhibitors of
metalloproteases (TIMPs). These inhibitors influence the
activation of the metalloproteases and act to modulate
proteolysis of ECM, notably during tissue remodelling and
inflammatory processes. There are also synthetic inhibitors
like marimastat for example, a butanediamide derivative
with a IC50 in the micromolar range. Inhibitors of MMPs
generally fall into three pharmacological categories :
collagen peptidomimetics and non-peptidomimetics,
tetracyclin derivatives and bisphosphonates.

MMPs inhibitors would therefore have the potential to
inhibit tumor growth by preventing local invasion and by
inhibiting tumor angiogenesis. The current existing non-
endogeneous inhibitors present the drawback of a poor oral
bioavailability as well as high cost. Moreover since these

are synthetic molecules, the synthesis route is generally cumbersome and necessitates purification step in order to recover functional mixtures.

5   Therefore it remains a need for a simple procedure for obtaining a metallo-proteinase inhibitory agent that would be cheap and easy to prepare and that could also be used in a food product or as neutraceutical food product.

10   It is also an object of the invention to provide a method for inhibiting metalloproteinases, preferably matrix-metalloproteinases.

     It is another object of the invention to provide an agent effective for inhibiting metalloproteinases,
15   preferably matrix-metalloproteinases.

     It is yet another objective of the present invention to provide a method for preparing an agent effective for inhibiting metalloproteinases, preferably matrix-metalloproteinases.

20   Accordingly, the present invention relates to a metallo-proteinases inhibitory agent, in particular a matrix-metalloproteinase' inhibitory agent, comprising casein-derived peptides obtained by hydrolysis of casein by a food grade bacteria.

25   The present invention also concerns the use of the inhibitory agent according to the present invention, systematically, locally or topically for inhibition of metalloproteinases, in particular matrix-metalloproteinases.

30   One other aspect of the present invention enable the manufacture of a metalloproteinases inhibitory agent, in

particular a matrix-metalloproteinases inhibitory agent, comprising the steps of :

- preparing a solution of casein and food grade bacteria,

5   - holding said solution under conditions effective to partially hydrolyse the casein to provide a metalloproteinases inhibitory agent, in particular a matrix-metalloproteinases inhibitory agent, containing casein-derived peptides,

10   - stopping the hydrolysis and

- optionnaly recovering the inhibitory agent from the solution.

The expression "food grade bacteria" refers to any bacteria that has not any harmful effect on human health or

15   that has the GRAS (generally recognized as safe) status. Such bacteria maybe selected from the group consisting of Lactobacilli and Bacilli. The preferred bacteria particularly suitable for the purpose of the process according to the present invention may be *Lactobacillus*

20   *helveticus* ssp, more particularly *Lactobacillus helveticus* deposited under the N°I-2864 at CNCM, Institut Pasteur, Rue du Dr Roux, Paris, on April 29, 2002.

An other aspect of the present invention relates to the manufacture of a metalloproteinases inhibitory agent,

25   in which isolated proteolytic enzymes of the preferred food grade bacteria are used instead or in combination with the bacteria.

A further aspect of the present invention relates to the manufacture of a fermented dairy product containing a

30   metalloproteinases inhibitory agent, preferably matrix-metalloproteinases inhibitory agent, comprising the steps of :

- contacting lactic acid bacteria with milk,
- fermenting the obtained mixture to obtain a fermented dairy product, under conditions to partially hydrolyse the casein of the milk into casein-derived peptides to provide a fermented dairy product containing a metalloproteinases inhibitory agent, in particular a matrix-metalloproteinases inhibitory agent.

It is also a further object of the present invention to provide inhibitors of metalloproteinases, in particular matrix-metalloproteinases, in the form of single-polypeptide-chain peptides. Thus, particularly preferred inhibitors according to the present invention are selected from the group consisting of the single-polypeptide-chain peptides set forth in SEQ ID N° 1 to 8, having the amino acid sequence:

E-N-L-L-R-F-F-V-A-P-F-P-E-V-F,
E-N-L-L-R-F-F-V-A-P-F-P-E-V-F-G-K-E-K-V,
N-E-N-L-L-R-F-F-V-A-P-F-P-E-V-F,
L-N-E-N-L-L-R-F-F-V-A-P-F-P-E-V,
E-N-L-L-R-F-F-V-A-P-F-P-E,
N-E-N-L-L-R-F-F-V-A-P-F-P-E,
G-L-P-Q-E-V-L-N-E-N-L-L-R-F-F and
F-V-A-P-F-P-E-V-F-G-K-E-K-V-N-E-L-S-K-D-I-G-S

or substantially homologous ones.
Other particularly preferred inhibitors according to the present invention are selected from the group consisting of the single-polypeptide-chain peptides set forth in SEQ ID N°9 to 16, having the amino acid sequence:

L-H-L-P-L-P-L-L
L-H-L-P-L-P-L-L-Q

N-L-H-L-P-L-P-L-L-Q

N-L-H-L-P-L-P-L-L

N-L-H-L-P-L-P-L-L-Q-S-W

D-V-E-N-L-H-L-P-L-P-L-L-Q-S-W

V-E-N-L-H-L-P-L-P-L

V-E-N-L-H-L-P-L-P and

L-P-L-L-Q-S-W-M-H-Q-P-H-Q-P-L-P-P-T-V-M-F-P-P-Q-S

or substancially homologous ones.

The expression "substantially homologous" as used here means a degree of homology in excess of 70%, preferably in excess of 80%.

The amino acids represented by the foregoing abbreviation are set forth in the description of the preferred embodiment.

The present invention also concerns the use of the inhibitors according to the present invention, systematically, locally or topically for inhibition of metalloproteinases, in particular matrix-metalloproteinases.

It is yet an other aspect of the present invention to provide pharmaceutical composition comprising an amount of inhibitors of metalloproteinases, in particular matrix-metalloproteinases, effective to reduce activities and/or functions of such metalloproteinases, in particular matrix-metalloproteinases.

Furthermore, other biologically active, improved analogs of the metalloproteinases inhibitors of the present invention

may be obtained by replacing various other amino acids in the inhibitor amino acid sequence.

In the present context, the term "metalloproteinase" embrass any kind of metal dependant proteolytic enzymes. Therefore, this term encompasses the matrix-metalloproteinases as described in the introduction of the present application such as MMP-1, MMP-2, MMP-3, MMP-4, MMP-5, MMP-6, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-13, MMP-14, MMP-15, MMP-16, MMP-17, MMP-18, MMP-19, MMP-20, MMP-21, MMP-22, MMP-23, MMP-24, MMP-25, MMP-26, MMP-27, and MMP-28. However, it also concerns other metal dependant proteinases like angiotensine converting enzyme (ACE), endothelium converting enzyme (ECE) and neutral endopeptidase since all these enzymes work according a similar chemical mechanism.

It has thus been surprisingly found that such agent shows a noticable proteolytic inhibitory effect regarding metallo-proteinases activity, and particularly regarding matrix metallo-proteinases activity. The present invention encompasses also the use of the inhibitors according to the present invention or composition comprising casein-derived peptides obtained by hydrolysis of casein by a lactic acid bacteria for the inhibition of metallo-proteinases, particularly matrix metallo-proteinases.

The inhibitors and inhibitory agent according to the present invention may be used for inhibition of metalloproteinases, particularly matrix-metalloproteinases, and therefore applied for inhibition of cancer invasion and metastasis, tissue ulceration, arthritis and periodontitis as well as inhibition of osteoclasts.

Such an agent is particularly suitable for use in food products, such as fermented and non fermented dairy products like yoghourts or fermented dairy beverages, for example.

Such agent may also be used in chewing-gum, candies or oral supplements, for example, for the local inhibition of metalloproteinases, in particular matrix-metalloproteinases, involved in periodontitis. In a similar way of use, the agent accoring to the invention may also be used in toothpaste or mouth-bath, for example, for the same purpose. The present agent may also be advantageously used in petfoods or treats for pets, for example. Indeed, a particular pet desease called Feline Osteoclastic Resorptive Lesion is suspected to be induced by local over-activity or activation of matrix-metalloprotease on the jaws of cats. The proteases thus activated induce the resorption of jaw-bone that will finally lead to loss of teeth and dental-incapacity of the pets, particularly cats. Therefore the local use of the inhibitors or inhibitory agent according to the present invention for reducing feline osteoclastic resorptive lesions presents a preferred aspect of the present invention.

Figure 1 describes peptide sequences from RP-HPLC subfractions 24-27 of fraction 6 from GPC showing a common amino acid sequence. Peptides from the candidate region retrieved in inactive fraction are shown in black whereas peptides characterised in subfractions twenty-four to twenty-seven and showing activity are shown in grey. **A** refers to Alpha-casein and **B** refers to Beta-casein.

Material and methods

HPLC/ Mass spectrometer

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- Finnigan Mat ion-trap LCQ mass spectrometer (Thermo Finnigan, San Jose, CA, USA)

- Finnigan Mat Spectra System P4000 (Thermo Finnigan, San Jose, CA, USA)

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- Finnigan Mat Spectra System AS3000 (Thermo Finnigan, San Jose, CA, USA)

- SEQUEST developed by John Yates and Jimmy Eng at the University of Washington and is protected by U.S. Patent 5,538,897

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- Agilent 1050 Series Modules and Systems for HPLC (Agilent Technologies, Palo Alto, CA, USA)

Colums for HPLC/MS

20

- Superdex 75 HR 10/30 (Amersham Pharmacia Biotech, Duebendorf, Switzerland)

- Column C8, 208TP54,pore size: 300 Å, particule size: 5μm, inner diameter: 4.6 mm, length: 250 mm (Vydac, Hesperia, California, USA)

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- Nucleosil C18, pore size: 100 Å, particule size: 5μm, inner diameter: 1mm, length: 250mm (Macherey-Nagel AG, Oensingen, Switzerland)

- Preparative C18, 218 TP152050, pore size: 300 Å, particule size: 10-20 μm, inner diameter: 50mm,

30

length: 250 mm (Vydac, Hesperia, California, USA).

Peptide synthesis

5   - ACT 348 Omega Multiple Peptide Synthesizer
       (Advanced ChemTech Inc, Louisville KY, USA)

Culture of lactic acid bacteria

10   Bacteria were grown without shaking, overnight in
       sterile MRS broth at 40°C in a water bath. UHT-skim
       milk was inoculated at 2% from the overnight MRS
       culture and fermentation occurred at 37°C. Bacteria
       were finally harvested as the pH of the fermented milk
15   lied below 4.5. The pH of the fermented milk was
       readjusted to 5.2 with NaOH and 2% of tri-sodium
       citrate were added to solubilize the caseins. The
       fermentation solution was allowed to rest for 10 min
       at RT.
20   Subsequently, bacteria were centrifuged at 3'700g, 4°C
       during 10 min, washed 3 times and resuspended in tris-
       maleate buffer.

            The optical density of the suspension was measured,
25   assuming that the increase in cell suspension
       turbidity of 1.0 unit of absorbance at 600 nm
       corresponds to 10^8 cells/ml.

       - *Buffer*: Tris-maleate (30 mM):   3.63 g
30   tris(hydroxymethyl)-aminomethane, 3.48 maleic acid

and 2.94 calcium chloride were added to 1 liter H_2O
and the pH was adjusted to 7.5 with NaOH.

5

Casein hydrolysis with lactic acid bacteria

The equivalent of OD=80 or $8*10^9$ cells per ml
(final conc.) bacteria were incubated at 37°C in a
10   water bath with sodium caseinate (4mg/ml) in 30 mM
tris-maleate buffer. Samples were taken as a function
of time (maximum: 5 hours). The proteolysis was
stopped by centrifugation at 14'000g and the
supernatant was recovered. The samples were conserved
15   frozen at -20°C for subsequent analyses.

- *Buffer*: Tris-maleate (30 mM):   3.63   g
tris(hydroxymethyl)-aminomethane, 3.48 maleic acid,
2.94 calcium chloride were mixed, the pH adjusted
20   with NaOH to 7.5 and the solution completed to 1
liter with H_2O.

Casein antigenicity ELISA test

25   Microtitration plates were coated with caseinate
in coating buffer at a concentration of 50 µg/ml and
incubated overnight at 4°C.
Four washing steps with phosphate buffered saline-
Tween-20 solution were performed before each
30   successive reagent addition. The unoccupied sites were

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blocked with fish gelatine buffer during 30 min. Test
samples dilutions and a standard solution were mixed
with rabbit anti-casein IgG-polyclonal antibodies
(dilution 1 : 10'000) and incubated for 1 hour at RT.

5   A peroxidase labelled second antibody (dilution 1 :
2'000) was then added and incubated for 30 min.
Subsequently, the reaction substrate (o-phenylene-
diamine, OPD) was added for 5-10 min and the reaction
stopped with 5% H_2SO_4 (final conc.). The plate was read

10   at 490 nm.

- *Coating buffer:* 795 mg Na_2CO_3 and 1.465 g $NaHCO_3$
  were added to 500 ml H_2O (pH 9.6).
- *PBS buffer:* 8 g NaCl, 200 mg KH_2PO_4, 200 mg KCl and

15   2.9 Na_2HPO_4 dodecahydrate were added to 1 liter H_2O.
- *PBS-Tween 20:* 0.25 g Tween-20 were added to 1 liter
  PBS.
- *Fish gelatine buffer:* 0.5% fish gelatine in PBS
  buffer.

20   - *Substrate buffer:* 0.51 g citric acid, monohydrate
  and 1.84 g Na_2HPO_4 were added to 100 ml H_2O. 15
  minutes before use 40 mg OPD, and finally 40 μl
  Perhydrol® were added.

25   <u>**Leucine aminopeptidase activity test**</u>

The leucine aminopeptidase activity was measured
according to the procedure described by Sarath et al
(*Sarath,G., R.De La Motte, and F.W.Wagner. 1989.*

30   *Protease assay methods. In Proteolytic enzymes: A*
practical approach. R.J.Beynon and J.S.Bond, editors.

IRL Press, Oxford. 25-56.) with some modifications. The assay was performed in a microtiter plate. In each well, 180µl of 100mM phosphate buffer, pH 6.5, 10µl enzyme solution and 10 µl H-Leu-pNA (10 mM in DMSO) were mixed and incubated at 37°C for 1 hour. The absorbance at 405 nm was measured as a function of time (molar absorption coefficient, $\varepsilon=10'500 \ M^{-1}cm^{-1}$).

Initial velocities are proportional to the enzyme concentration. The difference in absorbance ΔA_{400} is equivalent to the absorbance read during the enzymatic reaction minus the absorbance from the blank reaction (negative control).

The result is expressed in µmol p-nitroaniline formed per ml enzyme solution per minute [U/ml]

One leucine aminopeptidase unit (LAPU) is defined as the amount of enzyme required to liberate one micromole of p-nitroaniline per minute at 37°C.

Enzymatic hydrolysis of an active fraction from sodium caseinate hydrolysate

Fractions showing antiproliferative character were hydrolysed in a first step with 1% alcalase for 1 hour at 55°C in a thermomixer at 600 rpm. In a second step, the hydrolysate was incubated with 1% acid proteinase A for 1 hour at 50°C in a thermomixer at 600 rpm. The enzymatic reaction was stopped heating the sample 5 minutes at 95°C. Enzymes were finally removed from the

hydrolysate using Ultrafree®-0.5 centrifugal filter
units having a nominal molecular weight limit of
10'000 Da.

5   Percentages of enzyme given here represent the enzyme
proteinic content vs. the hydrolysate proteinic
content.

10   **Culture of human intestinal epithelial cells (IECs) and
proliferation assay**

- Human colonic adenocarcinoma cell line HT29 (ATCC:
  HTB-38) from the American Type Culture Collection
  (ATCC, Manassas, VA).

15   - DMEM, high glucose (4.5 g/l), with stable glutamine
  (BioConcept, AMIMED, Allschwil, Switzerland)

Undifferentiated cells (passages 45-78) were
maintained in glucose-containing DMEM supplemented
20   with 10% FCS at 37°C in a 5% CO_2/air incubator. Culture
medium was changed every two days until the cell
monolayers reached 50% confluence. For stimulation
assays, HT-29 cells were plated at $1.2 * 10^4$ cells/well
in 96-well flat-bottom plates. After incubation for 4
25   days, the amount of cells present in the wells before
incubation with the samples was assessed adding
neutral red solution in 4 wells and incubating the
plate for 45 min. The HT-29 cells were then washed
twice with serum-free media (inclusive the wells with
30   neutral red solution). The wells coloured with neutral
red remained from now on free of any media.

HT-29 cells were then incubated 24 hours respectively
in the presence of 80 µg (hydrolysed) sodium caseinate
samples in 200 µl DMEM, in 200 µl serum-free medium
(blank), in 200 µl medium with serum (positive
control) or in 0.9% saline solution (negative
control). After incubation, the cells were washed
twice with serum-free media, neutral red solution was
then added and the cells incubated for 45 min. All
wells were washed three times with saline water and
residual solution was removed hitting the plate gently
on a tissue. The dye was finally extracted with 100 µl
of a extraction solution and the optical density
measured at 540 nm. The optical density measured
correlates with the number of cells present in the
wells.

The proliferation was calculated by the difference in
absorbance between the cells having been incubated 24
hours with sodium caseinate samples, with serum-free
medium, with medium containing serum or with 0.9%
saline solution and the cells before incubation.
The difference in absorbance ΔA_{540} from the blank
(cells incubated with serum-free medium) was
normalized. The results were then expressed as
percentage with respect to the blank.

- *Culture medium with serum*: DMEM was supplemented
  with 10% decomplemented FCS and 1% Penicillin-
  Streptomycin. The solution was sterile filtered Ø
  22 µm.

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- *Culture medium, serum-free*: DMEM was supplemented with 1% Penicillin-Streptomycin. The solution was sterile filtered 22 μm.
- *Extraction solution*: 1% acetic acid, 50% EtOH in water.
- *Neutral red stock solution*: 617 mg of Neutral Red were dissolved 100 ml in 0.9% NaCl solution.The solution was autoclaved at 120°C for 15 min and stored at 4°C in light –shielded bottles.
- *Neutral red solution*: Dilute 3:10 Neutral Red stock solution in DMEM, serum-free. Use this solution for the assay.

Foetal calf serum (FCS) was decomplemented during 30 min at 56°C.

Lactate dehydrogenase cytotoxicity test

Cell viability was rigourously measured according the manufacturer's recommendations (Roche Diagnostics). The test is based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells in the supernatant.

Hydrolysate fractionation by gel permeation

Samples were analysed using a Superdex 75 column with an isocratic elution using 50 mM ammonium acetate, pH 7, as solvent for 65 min. The flow rate was 0.5 ml/min, at pressure of 17-19 bar. Detection was accomplished at 280 and 215 nm.

Prior separation, the samples were heated for 5 min at 90°C and centrifuged at 14'000g in a bench centrifuge. 100 µl supernatant were then injected for analysis and

5   separation. Fractions were collected as a function of their molecular weight.

Finally, the collected fractions were lyophilised and resuspended in H_2O twice before final lyophilisation. This procedure should ensure the disappearance of any

10   traces of ammonium acetate.

HPLC-solvents were sterile-filtered (Ø 22µm) and sonicated at least 5 minutes to remove any air bubbles.

15

Hydrolysate fractionation by reverse-phase chromatography, using a C8 column

20   Since 100 µl caseinate hydrolysate had been fractionated on the Superdex75 column, the resulting lyophilised gel permeation fractions were resuspended in 100 µl H_2O for testing the inhibition of of cell proliferation . The concentration of each peptide in a

25   fraction was thus comparable to the concentration of the peptide in the initial hydrolysate sample.

Samples (100 µl) with a molecular weight between 5'000 and 1'000 Da were analysed using a C8-column

30   with a linear gradient increase of solvent B in solvent A as follows: 5 minutes isocratic elution at 0%B, 0-50% B in 60 minutes, 50-100% B in 1 minute, 4

minutes at 100% B, change to 100%A in one minute and finally re-equilibration at 100%A during 9 minutes.

Samples (100 µl) with a molecular weight lower than 1'000 Da were analysed using a C8-column with a linear gradient increase of solvent B in solvent A as follows: 5 minutes isocratic elution at 0%B, 0-35% B in 60 minutes, 35-100% B in 1 minute, 4 minutes at 100% B, change to 100%A in one minute and finally re-equilibration at 100%A during 9 minutes.

Total time run for both analyses was 80 min and the flow rate amount to 0.8 ml/min with a pressure of 105-120 bar. The injection volume was 100 µl. Following chromatographic separation, fractions were collected every 2.5 min (2 ml volume). The collected fractions were lyophilised and resuspended in H_2O twice before final lyophilisation. This procedure should ensure the disappearance of any traces of trifluoroacetic acid. Finally, the lyophilised fractions were resuspended in 100 µl H_2O to ensure that the concentration of each peptide before fractionation is the same after fractionation.

HPLC-solvents were sterile-filtered (Ø 22µm) and sonicated at least 5 minutes to remove any air bubbles.
- *Solvent A*: 0.05% TFA in water
- *Solvent B*: 0.045% TFA, 80% acetonitrile in water

ESI-MS/MS

Peptides separated by GPC methods were characterised using a SpectraSystem HPLC with a C8 or a C18 column coupled to the Finnigan LCQ ion trap mass spectrometer equipped with an ESI source. The HPLC system consisted of a quaternary pump (TSP P4000), an autosampler (TSP AS3000) and a UV/VIS detector (UV/VIS 205 from Linear Instruments) equipped with a high pressure flow-cell (1.6 µl volume, 2 mm pathlength). HPLC flow was directed via 0.005'' ID PEEK tubing to a micro flow-splitter with 10% going to the MS and 90% being collected in a fraction collector. The MS instrument was tuned and calibrated using the manufacturer's protocols (ThermoFinnigan). The ESI source was operated at 4.5 kV and the interface capillary heater was set to 200°C. Sheath gas flow was maintained at flow rates of 50 ml/min.

All spectra were obtained in positive mode and recorded at unit-mass resolution. Automated MS/MS spectra were acquired with relative collision energy for CID preset at 35% and an isolation width of 1 m/z units. During an automated run, if an ion was present in a scan above a specified threshold, a product ion spectrum was acquired. After two product ion spectrum scans of the most abundant ion, it was excluded to trigger further MS/MS scans ("dynamic exclusion" option in Xcalibur software package). The second most abundant ion, if still above specified threshold, could trigger again product ion spectrum acquisition.

Dynamic exclusion allowed the generation of MS/MS
spectra of peptide mixtures in peaks not fully
resolved by chromatography. The mass spectrometer
continued to alternate between MS and MS/MS mode until
5   the ion intensity dropped below the threshold. The
scan range was set at m/z 150.0-2000.0 .

Data analysis

Full scan MS and MS/MS data acquisition and
10   analysis was performed with Xcalibur software V1.0 SR1
(Finnigan), including Bioworks V1.0 software package
for SEQUEST™ database searches.
Experimentally obtained peptide masses were compared
to SwissProt protein database using *PeptIdent* (Peptide
15   Mass Fingerprinting) and *PeptideMass* programs
accessible from the ExPASy Molecular Biology Server of
the Swiss Institute of Bioinformatics at the Internet
site http://www.expasy.ch.
MSMS spectra analysis was performed using the SEQUEST™
20   program. It correlates uninterpreted MSMS spectra of
peptides with amino acid sequences from protein and
nucleotide databases. Based on this correlation it
determines the amino acid sequence and thus the
protein(s) that correspond to the mass spectrum being
25   analysed. More information can be obtained at the
SEQUEST™   Internet   site
http://thompson.mbt.washington.edu/sequest.

Peptide synthesis procedure

30

All peptides (400-500 mg) were synthesised on an
Advanced Chemtech peptide synthesiser ACE 348 omega.

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The α-amino function of the amino acids was protected with the base labile fluorenyl methoxy carbonyl (Fmoc) and side chains of tri-functional amino acids with acid labile protecting groups, i.e. Arg(Boc)2, Glu(Otbu), His(Trt) and Asn (Mtt).

In general, prior to peptide synthesis, the resin was swollen in dichloromethane (DCM) during 30 min. Peptides were assembled by sequential amino acid coupling on Sasrin resin applying the Fmoc-strategy [180]. According to the published procedures of automated peptide synthesis, a deprotection /coupling cycle comprises I) a 10 min deprotection of Fmoc amino acid derivative (2 eq) in DMF with Benzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP, 2.5 eq) as coupling reagent and N,N-diisopropyl ethylamine (DIEA, 5 eq) as base.

Cleavage of the peptide from the resin was performed with 2% trifluoroacetic acid (TFA) in DCM during 20 min. After treatment, the resin was separated by filtration and the filtrate concentrated in vacuo. For complete removal of the side chain protection, the peptide was treated with a cocktail of TFA/Triisopropylsilan (TIS)/water (90:5:5, v/v). After 1hour, cold diethyl ether was added to precipitate the product. The precipitate was collected by centrifugation and lyophilised from a water/acetonitrile (1:1, v/v) solution to afford a colourless solid.

After lyophilisation, the crude product was purified by preparative RP-HPLC on a C18-column applying a linear gradient from 20%A to 60%A (A: 0.9% TFA in acetonitrile, B: 10% A in H2O) over 30 min at

flow rate of 80 ml/min. The eluate was monitored by UV absorption at 214 nm wavelength, appropriate fractions were collected and lyophilised. The peptide was characterised by electrospray ionisation mass spectrometry (ESI-MS) and analytical RP-HPLC.

Angiotensin-converting-enzyme inhibitory test

This procedure is a rapid spectrophotometric method utilising the synthetic tripeptide substrate N-[3-(2-furyl)acryloil]-L-phenylalanylglycylglycine   (FAPGG). K_m-value: 0.3 mM.

FAPGG was hydrolysed to furylacryloylphenylalanine (FAP) and glycylgylcine. Hydrolysis of FAPGG resulted in a decrease in absorbance at 340 nm.
The ACE reagent and the ACE Calibrator were reconstituted according to the manufacturer's recommendations.

The assay was performed in quartz cuvets. 0.05 ml ACE Reagent Solution, 0.05 ml casein hydrolysate and 0.05 ml ACE calibrator were mixed and incubated at 37°C for 10 minutes.
The absorbance at 340 nm was measured as a function of time.

The ACE activity in the sample was determined by comparing the sample reaction rate to that obtained with the ACE calibrator. The results are expressed as percentage with respect to the control.

- 23 -

Matrix metalloproteinase inhibitory test

The assay was performed in microtiter plates. In
each single well 1 µl enzyme, the inhibitor at the
5   desired concentration and finally 5 µl substrate were
added to PBS buffer (pH 7.0-7.5), final volume: 200
µl. The reaction mixture was incubated at 37°C for 2
hours. The increase in fluorescence was continuously
recorded, exciting at 485 nm and reading the emission
10   at 530 nm, with a gain of 40.

- *Inhibitors*:   -   o-phenantroline: 9.9 mg in 25
                        µl DMF (dimethylformamide) → add 1 µl
                        (final concentration: 10 mM)

15   -   synthetic inhibitors at different
                        concentrations

- *Substrate*:   DQ-gelatine: 1 mg/ml in PBS → add
                        5 µl to the reaction (reaction volume:
20   200 µl) (final concentration: 25 mM)

- *Enzymes*:   -   MMP-7: stock solution: 1 U/µl
                    -    MMP-2: stock solution : 0.05 µg/µl
                    -    MMP-9: stock solution: 0.05 µg/µl

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Results & Discussion

Generation of the peptide library with *Lb. helveticus* CNCM I-2864 ......

Following preliminary trials with several LAB representing the variety of LAB used in dairy products, the *Lb. helveticus* NCC 619 strain was selected to hydrolyse sodium caseinate in a buffered system. Caseinate hydrolysis occurred at 37°C for 5 hours. Samples were taken as a function of the time and proteolysis was stopped by centrifugation of the bacteria and recovery of the supernatant. The caseinate hydrolysates taken at different times constituted the peptide libraries studied below.

Leucine aminopeptidase test

This test was performed to check that caseinate hydrolysis was the action of the extracellular LAB proteinase and not the consequence of peptidases' activities. LAB display a unique cell wall associated proteinase which is performing the first step in the degradation of proteins. However, many peptidases are located in the cytosol of the bacteria and membrane leakage could be responsible for their release in the fermentation medium.

The conversion of leucine p-nitroaniline to p-nitroaniline catalysed by leucine aminopeptidase was measured in the samples that were previously analysed by SDS-PAGE and ELISA.

This analysis demonstrated that little aminopeptidases were released from the cytosol of the bacteria into the medium during caseinate hydrolysis.

The overall hydrolysis of caseins by aminopeptidases was relatively low, reaching approximately 1% after 5 hours fermentation. Hydrolysis of caseins was thus resulting quasi exclusively from the serine-type extracellular bacterial proteinase.

Characterisation of the peptide library

The casein-derived peptide library generated by *Lb. helveticus* CNCM I-2864 …. was characterised using liquid chromatography in combination with mass spectrometry.

Fractionation by HPLC

The strategy chosen for the fractionation of caseinate hydrolysates was set up in two steps.
A first fractionation occurred on a molecular weight basis by gel permeation chromatography. The second fractionation was based on the hydrophobic interactions of the casein-derived peptides with a C_8-reverse phase chromatography column.

♦   **Superdex 75 gel permeation chromatography (GPC)**

With a molecular weight standard, retention times were experimented on the Superdex 75 .

Finally, seven fractions were collected after separation of the caseinate hydrolysate using size exclusion chromatography.

Fraction's name	F^{ion} 1	F^{ion} 2	F^{ion} 3	F^{ion} 4	F^{ion} 5	F^{ion} 6	F^{ion} 7
Retention time [min]	13.8 – 15.2	15.2 – 16.7	16.7 – 20.5	20.5 – 26.4	26.4 – 32.2	32.2 – 39.0	39.0 – 65.0
Molecular weight range [Da]	45'000 – 40'000	40'000 – 35'000	35'000 – 25'000	25'000 – 15'000	15'000 – 5'000	5'000 – 1'000	<1'000

Table 1 : Result of the first fractionation step achieved with Superdex 75 gel permeation chromatography (GPC)

♦ **C8-reverse phase chromatography**

Fractions 6 (5'-1'ooo Da) and 7 (<1'000 Da) from GPC were further fractionated using reverse-phase chromatography.

Reverse-phase chromatography was used in combination with mass spectrometry for the characterisation of the sample. The HPLC flow was directed to a micro flow-splitter with 10% going to the MS and 90% being collected in a fraction collector. Mass spectrometric analysis and collection of fractions were thus performed in the same run, allowing the determination of the exact qualitative peptidic composition of each collected fraction.

Thirty-two fractions were collected as a function of the time, every 2.5 min.

Reverse-phase separation led to the identification of around five hundred peptides. All peptides were analysed and characterised using mass spectrometric analysis and in particular MS/MS data.

Mass-spectrometric characterisation of the low molecular weight peptide fractions of the hydrolysates

Reverse-phase HPLC in conjunction with ESI-MS detection of casein-derived peptides in fractions 6 and 7 from GPC resulted in the identification of more than five hundred peptides.

Fragmentation patterns of peptides were obtained by MS/MS analysis of the most intense ions. To perform protein identification, mass spectrometry fragmentation patterns were used to search protein database. This work was done using the SEQUEST browser ® software. This tool was able to convert the character-based representation of amino acid sequences in a protein database to fragmentation patterns that were compared against the MS/MS spectrum generated on the target peptide.

The algorithm initially identified amino acid sequences in the database that matched the measured mass of the peptide, compared fragment ions against the MS/MS spectrum, and generated a preliminary score for each amino acid sequence. A cross correlation analysis was then performed on the top 500 preliminary scoring peptides by correlating theoretical,

reconstructed spectra against the experimental spectrum. Best scores and the corresponding peptides were then proposed.

5   Fragmentation pattern showed predominance of the products ions at m/z 1053.3 and at m/z 730.8. These two intense signals correspond to fragments issued from internal cleavage near the proline; the strongest signal corresponding to the extension from the proline

10   in the direction of the C-terminus. Moreover, we saw generation of both the b-type (b_2; m/z 292.2, b_3; m/z 421.4, b_5-b_7; m/z 617.7, 730.8, 787.9, b_9-b_{11}; m/z 984., 1140.3, 1197.4 and b_{13}; m/z 1441.7) and y-type (y_3-y_{14}; m/z 342.5, 489.6, 586.8, 643.8, 800.0, 899.1,

15   996.2, 1053.3, 1166.5, 1256.6, 1362.7, 1491.8) ions. These types of ions are predominantly generated in low energy collision induced dissociation.
All these informations demonstrate the validity of the proposed peptide sequence. It was processed in a

20   similar way for all characterised peptides. Fragmentation patterns with no clear assignable b- or y-type ions were rejected.
The characterization of the peptide library using mass spectrometry allowed the identification of five

25   hundred low molecular weight peptides
In conclusion, we have the confirmation that the *Lb. helveticus* NCC 619 strain is able to generate peptides displaying bioactivity, as shown in the table above. This strain generated also more than five thousand low

30   molecular weight peptides, all characterised by mass spectrometry. This peptide library, including peptides

from two to twenty-eight amino acids, displayed also a
new bioactive potential, as will be shown later.

The peptide library generated in this work is not as
wide as a random synthetic peptide library or even as
a phage display library. The identified peptides
however cover the complete protein sequence of the
caseins. Proteolytic peptide libraries obtained with
enzymes that show a broad specificity have the
advantage to generate peptides of different length
with overlapping amino acid sequences, thus increasing
the chances of positive hits.

Biological activity of caseinate hydrolysates

Cellular proliferation tests

The effect of caseinate hydrolysate on human colon
cancer cell line HT-29 was investigated. This
experimental set-up provides an experimental model in
which it is possible to study, directly on the cells,
the effects of fermented milk or fermented milk
fractions.

For the first screening, HT-29 cells were incubated
24 hours with caseinate hydrolysates fermented for
one, two and five hours with *Lb. helveticus* CNCM I-
2864 (*Table 2*). All assays were performed in
triplicate. Cell number was determined before and
after incubation with caseinate hydrolysate, allowing

the distinction between inhibition of cell proliferation and induction of cell death.

The observed cell growth of HT-29 in cellular culture medium without serum was normalised. As positive control, cells were incubated in presence of foetal calf serum, this serum containing various growth factors. Finally, as negative control, cells were incubated 24 hours in physiological saline, without any cellular culture medium. After 24 hours incubation with the samples, the cells were dyed using neutral red.

Indeed, the supravital stain neutral red is weakly cationic and is thus actively concentrated by viable cells in intracytoplasmic vacuoles and granules by binding to anionic sites. It has been described as a convenient and reproducible assay to determine cell proliferation in 96-well plates.

	+ serum	– serum	0.9% NaCl	Caseinate	Tris maleate	CNCM I-2864 0h	CNCM I-2864 1h	CNCM I-2864 2h	CNCM I-2864 5h
% prolif	112 +/-17	100 +/- 9	-26 +/- 8	93 +/- 12	72 +/- 13	83 +/- 5	23 +/- 5	26 +/- 3	104 +/- 10

Table 2 : Effect of caseinate hydrolysates on growth of HT-29 cells. Results are expressed as percentage of OD (OD measured at 540 nm) with respect to the control (-serum).

As complementary controls, it was checked that neither sodium caseinate, the initial substrate, nor tris-maleate, the buffer used during caseinate hydrolysis had any negative effect on HT-29 cellular proliferation.

As we see from Table 2, sodium caseinate as well as tris-maleate had no activity towards cellular proliferation.

However, caseinate hydrolysates fermented for one and two hours showed high antiproliferative activity. This activity disappeared after 5 hours hydrolysis.

This particular feature indicates that some antiproliferative factors were generated during caseinate hydrolysis. They were then at their turn digested, and lost their bioactivity, explaining the disappearance of the antiproliferative properties of the caseinate hydrolysate at five hours fermentation. Thus antiproliferative factors present in the hydrolysates are probably of proteinic origin.

This hypothesis was examined (Table 3). A fraction showing antiproliferative activity was tested in parallel with the same fraction having been digested successively by alcalase and proteinase A, two commercially available enzymes.

	+ serum	- serum	0.9% NaCl	Caseinate	CNCM I-2864 1h	CNCM I-2864 1h, ΔT	(E,ΔT) + CNCM I-2864 1h	(CNCM I-2864 1h+E) ΔT
% prolif	121 +/- 4	100 +/-8	5 +/- 5	93 +/- 7	28 +/- 12	13 +/- 12	31 +/- 6	100 +/- 13

Table 3 : Effect of caseinate hydrolysates on growth of HT-29 cells. Results are expressed as percentage of OD (OD measured at 540 nm) with respect to the control (-serum).
CNCM I-2864, 1h : caseinate hydrolysate fermented for one hour with the Lb.helveticus CNCM I-2864 for one hour; CNCM I-2864 1h, ΔT: heat-treated caseinate hydrolysate; (E,ΔT) CNCM I-2864 1h: non-treated caseinate hydrolysate with addition of heat-inactivated enzyme; (CNCM I-2864 1h + E) ΔT: caseinate hydrolysate further hydrolysed with enzymes and finally heat-treated for enzyme inactivation.

The data presented in Table 2 show the effect on the proliferation of the colon cancer cell line HT-29 of an active fraction which has been further digested by commercially available enzymes. The enzymes were inhibited by heating.

In this specific experiment, the caseinate fraction fermented for one hour with *Lb. helveticus* CNCM I-2864. showed a reproducible inhibiting effect with 72% (previously 77%) inhibition of proliferation (*Table 2* and *Table 3*).

The same fraction, that had undergone further enzymatic treatment with alcalase and proteinase A, lost its antiproliferative character.

In order to establish the validity of this result, we checked that the final heating step was not responsible for the loss of bioactivity.

If we make the assumption that the responsible factor is of proteinic origin, heating the sample could induce a change in the conformation of active proteins, leading to the non-recognition of the mentioned protein by a putative receptor involved in cellular proliferation mechanism. Thus, the active fraction was submitted to heating, without enzymatic treatment. The effect on cellular proliferation of this heated fraction was in all points similar to the ones of the non-heated hydrolysate. To reject any interference of the heat-inactivated enzymes in the test, the active fraction was mixed with heat-inactivated enzymes and the activity of the sample remained unaffected.

At this stage, we were not able to differentiate between an active proteinic factor generated by the lactic acid bacteria strain during caseinate hydrolysis or a casein-derived peptide. This differentiation will be made when mass spectrometric analysis of the samples will be performed.

Casein-derived hydrolysates are not cytotoxic

To be sure that the antiproliferative effect observed on the human colon cancer cell line was not the observation of the induction of necrosis, cytotoxicity tests were performed. Most current assays for measuring cytotoxicity are based on alterations of plasma membrane permeability and the consequent

release of cytoplasmic enzymes or the uptake of dyes, normally excluded by viable cells. We here chose to measure the lactate dehydrogenase (LDH) activity released from damaged cells (Table 4).

	Lysate	+ serum	– serum	Casei nate	Tris malea te	CNCM I- 2864 0h	CNCM I- 2864 1h	CNCM I- 2864 2h	CNCM I- 2864 5h
% prolif	100 +/-8	4 +/-2	0 +/-6	9 +/-3	-1 +/-4	-6 +/-6	-4 +/-7	-6 +/-3	-7 +/-3

Table 4 : Determination of the cytotoxic potential of caseinate hydrolysates on HT-29 cells. Results are expressed as percentage of OD (OD measured at 490 nm) with respect to the control (lysate).

No LDH activity was detected in any culture media of cells incubated 24 hours with caseinate hydrolysates. Based on this test, we may affirm that sodium caseinate hydrolysates did neither alter cell permeability nor induced necrosis.
Little LDH activity was however observed when cells were incubated in physiological saline only, showing that slight membrane alterations occurred as the result of harsh culture conditions.

Thus, considering these last results, we knew that sodium caseinate hydrolysates inducing a decreased colon cancer cell line proliferation were not cytotoxic.

Inhibition of cell proliferation by GPC fractions

Considering the results of the first proliferation test, we have seen that hydrolysates fermented for one and two hours with *Lb. helveticus* CNCM I-2864 had antiproliferative character.

In Table 5 we present the cellular growth experienced when testing fractions of a caseinate hydrolysate fermented for two hours and separated on a molecular weight basis.

	+ serum	- serum	0.9 % NaCl	F1	F2	F3	F4	F5	F6	F7	C2 rec.
% prolif	122 +/- 9	100 +/- 13	13 +/- 9	97 +/ -7	108 +/- 11	95 +/- 15	102 +/- 19	104 +/- 19	54 +/- 8	62 +/- 12	29 +/- 10

Table 5 : Effect of caseinate hydrolysate's gel chromatography fractions (two hours fermentation) on growth of HT-29 cells. Results are expressed as percentage of OD (OD measured at 540 nm) with respect to the control (-serum).
F1:45'-40'ooo Da peptides; F2: 40'-35'000 Da; F3: 35'-25'000 Da peptides; F4: 25'-15'000 Da peptides; F5: 15'-5'000 Da peptides; F6: 5'-1'000 Da peptides; F7: peptides >1'000 Da.C2 Reconst: fraction reconstituted on the basis of F1-F7.

Fractions F1 to F5 from GPC represent peptides with molecular weights ranging from 45'-5'000 Da. These fractions did not contain any antiproliferative factors or these were present in insufficient quantity for detection.

On the contrary, F6 from GPC (corresponding to peptides: 5'-1'ooo Da) and F7 from GPC (peptides <1'ooo Da) showed important antiproliferative activities and were further investigated.

We notice moreover that isocratic fractionation using neutral ammonium acetate solution is a mild technique ensuring biological activity's preservation. Indeed, we did not loose biological activity through separation of the hydrolysate as a reconstituted fraction (C2 Reconst.) showed identical activity as before separation (Table 4).

Samples were analysed by mass spectrometry. We knew the exact peptidic content of each subfraction. Fractions showing antiproliferative potential, but inactive fractions too were characterised using mass spectrometry. Thus we searched for a peptidic sequence present in all slightly active subfractions derived from a sample having shown antiproliferative activity, but absent in a sample not having exhibited bioactivity.

This search was effected and the results are presented in Figure 1. Two potential candidates were identified, one sequence was derived from alpha-S1 and the other from beta casein.

Peptides from the candidate region retrieved in an inactive fraction are shown in black whereas peptides characterised in subfractions twenty-four to twenty-seven and showing activity are shown in grey.

Decision was taken to synthesize chemically the candidate active regions.

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Peptide synthesis

Peptides sequences VENLHLPLPLL, ENLHLPLPLL, NLHLPLPLL and LNENLLRFFVAPFPEVFG, NENLLRFFVAPFPEVFG, ENLLRFFVAPFPEVFG, FVAPFPEVFG were chemically synthesised by Dr. A. Razaname at the EPFL, Lausanne using the Fmoc-solid phase peptide synthesis strategy.

It was for chemical practical purposes that the alpha-S1 casein sequence was elongated at the C-terminus from phenylalanine to glycine.

Inhibition of proliferation by synthetic peptides

Synthetic peptides that were tested on HT-29 cell line confirmed their antiproliferative potential.

The tested peptide LNENLLRFFVAPFPEVFG (α_{S1} 31-48) strongly inhibited proliferation (Table 6). The same experiment was performed with a shorter version of the

former mentioned peptide: ENLLRFFVAPFPEVFG (α_{S1} 33-48).
Best antiproliferative results were obtained at two to
four micromolar concentrations, giving an
antiproliferative pattern similar to the one observed
for LNENLLRFFVAPFPEVFG (data not shown). On the basis
of this data and for this particular sequence, N-
terminus elongation was shown to preserve biological
activity.

Peptide concentration [μM]	0	133	66	33	17	8	4	2
% prolif	100	135	119	131	114	160	44	56

Table 6 : Effect on growth of HT-29 cells of the
alpha-S1 derived synthetic peptide: LNENLLRFFVAPFPEVFG
(α_{S1} 31-48). Results are expressed as percentage of
counts with respect to the control.

In vitro inhibition of MMPs

We chose to focus attention on MMP-2, -7 and -9.
First, MMP-9 is involved in breast, colorectal, lung,
prostate, pancreatic and ovarian tumors. Its
expression is closely associated with the conversion
from radial growth phase to vertical growth phase and
subsequent metastasis. Secondly, MMP-2 is involved in
breast, colorectal, lung, prostate, pancreatic and
ovarian tumors. Its increasing expression indicates
progressing tumor grade. Finally, MMP-7 is involved in

breast, colorectal, lung and prostate tumors. MMP-7 has been implicated in the early steps of tumor development.

Both families of synthetic peptides were tested for their potential as MMP inhibitors: the four peptides derived from casein alpha-S1 and the three derived from beta caseins.
As positive control we tested o-phenantroline, a broad metalloproteinase inhibitor, which showed complete MMP inhibition at 10 mM concentration (data not shown) Inhibition data concerning MMP-2, MMP-7 and MMP-9 inhibition (Table 7) showed increasing inhibiting activities with increasing N-terminal length of peptides derived from beta casein.

Concerning MMP-2 and MMP-7, we observe an increasing inhibitory potential with N-terminal elongation of the ENLLRFFVAPFPEVFG sequence. However, this tendency is not confirmed in the case of MMP-9. Moreover, FVAPFPEVFG showed an equal inhibitory potential for all three MMPs tested.

In general, we might observe relatively large IC_{50} values differences between MMP-7 and MMP-9 or MMP-2. These differences might be explained by the structural variation between these enzymes. In fact, MMP-7 is lacking the hemopexin-like domain. This domain has been shown to play a functional role in substrate binding and/or in interactions with the tissue inhibitors of metalloproteinases (TIMPs), a family of specific MMP protein inhibitors.

		IC$_{50}$ values [µM]		
		MMP-2	MMP-7	MMP-9
Derived from beta casein	NLHLPLPLL	450	1200	600
	ENLHLPLPLL	200	275	300
	VENLHLPLPLL	150	250	300
Derived from alpha-S1 casein	FVAPFPEVFG	500	300	400
	ENLLRFFVAPFPEVFG	425	1000	150
	NENLLRFFVAPFPEVFG	275	850	50
	LNENLLRFFVAPFPEVFG	250	300	150

Table 7 : IC50 [µM] for the inhibition of MMPs using a fluorescein conjugated gelatin

In vitro inhibition of ACE

It is important to notice that ACE belongs to the zincin family of proteinases containing the *HEXXH* short binding consensus sequence, the family including matrix metalloproteinases too.

Inhibitors of matrix metalloproteinases might thus interfere with other zinc-metalloproteinases. We decided to test MMP-inhibitory peptides for their ACE-inhibitory potential.

		IC$_{50}$ values [µM] ACE
Derived from beta casein	NLHLPLPLL	15
	ENLHLPLPLL	250
	VENLHLPLPLL	175
Derived from alpha-S1 casein	FVAPFPEVFG	650
	ENLLRFFVAPFPEVFG	250
	NENLLRFFVAPFPEVFG	55
	LNENLLRFFVAPFPEVFG	280

Table 8 : IC50 [µM] for the inhibition of ACE using the synthetic tripeptide substrate N-[3-2(furyl)acryloyl]L-phenylalanylglycylglycine.

From the data of Table 7 and that of Table 8 we notice a similar behaviour of alpha-S1 derived peptides concerning the inhibition of both ACE and MMP-9.
An identical behaviour for the inhibition of MMP-9 and ACE was displayed by peptides derived from the beta casein sequence.

CLAIMS

1. Metallo-proteinases inhibitory agent, in particular a
matrix-metalloproteinases inhibitory agent, comprising
casein-derived peptides obtained by hydrolysis of casein by
a food grade bacteria.

2. Process for the manufacture of a metalloproteinases
inhibitory agent, in particular a matrix-metalloproteinases
inhibitory agent, comprising the steps of :
    - preparing a solution of casein and food grade
bacteria,
    - holding said solution under conditions effective to
partially hydrolyse the casein to provide a
metalloproteinases inhibitory agent, in particular a
matrix-metalloproteinases inhibitory agent, containing
casein-derived peptides,
    - stopping the hydrolysis and
    - optionnaly recovering the inhibitory agent from the
solution.

3. Process according to claim 2, characterized in that the
food grade bacteria is selected from the group consisting
of Lactobacilli and Bacilli.

4. Process according to claim 2, characterized in that the
food grade bacteria is selected from the group consisting
of *Lactobacillus helveticus* ssp, and *Lactobacillus
helveticus* CNCM I-2864.

5. Process according to claim 2, characterized in that isolated proteolytic enzymes of the preferred food grade bacteria are used instead of the bacteria or in combination with the bacteria.

5

6. Process for the manufacture of a fermented dairy product containing a metalloproteinases inhibitory agent, preferably matrix-metalloproteinases inhibitory agent, comprising the steps of :

10   - contacting lactic acid bacteria with milk,
          - fermenting the obtained mixture to obtain a fermented dairy product, under conditions to partially hydrolyse the casein of the milk into casein-derived peptides to provide a fermented dairy product containing a

15 metalloproteinases inhibitory agent, in particular a matrix-metalloproteinases inhibitory agent.

7. Inhibitors of metalloproteinases, in particular matrix-metalloproteinases, selected from the group consisting of

20 the single-polypeptide-chain peptides set forth in SEQ ID N° 1 to 8, having the amino acid sequence:

E-N-L-L-R-F-F-V-A-P-F-P-E-V-F,

E-N-L-L-R-F-F-V-A-P-F-P-E-V-F-G-K-E-K-V,

25   N-E-N-L-L-R-F-F-V-A-P-F-P-E-V-F,

L-N-E-N-L-L-R-F-F-V-A-P-F-P-E-V,

E-N-L-L-R-F-F-V-A-P-F-P-E,

N-E-N-L-L-R-F-F-V-A-P-F-P-E,

G-L-P-Q-E-V-L-N-E-N-L-L-R-F-F and

30   F-V-A-P-F-P-E-V-F-G-K-E-K-V-N-E-L-S-K-D-I-G-S

8. Inhibitors of metalloproteinases, in particular matrix-metalloproteinases, selected from the group consisting of the single-polypeptide-chain peptides set forth in SEQ ID N°9 to 16, having the amino acid sequence:

L-H-L-P-L-P-L-L

L-H-L-P-L-P-L-L-Q

N-L-H-L-P-L-P-L-L-Q

N-L-H-L-P-L-P-L-L

N-L-H-L-P-L-P-L-L-Q-S-W

D-V-E-N-L-H-L-P-L-P-L-L-Q-S-W

V-E-N-L-H-L-P-L-P-L

V-E-N-L-H-L-P-L-P and

L-P-L-L-Q-S-W-M-H-Q-P-H-Q-P-L-P-P-T-V-M-F-P-P-Q-S

9. Use of the inhibitory agent according to claim 1 for systematical, local or topical inhibition of metalloproteinases, in particular matrix-metalloproteinases.

10. Use of the inhibitors according to claims 7 and 8 for systematical, local or topical inhibition of metalloproteinases, in particular matrix-metalloproteinases.

11. Use of inhibitors according to claims 7 and 8 or inhibitory agent according to claim 1, in toothpaste, mouth-bath or food products, such as fermented and non fermented dairy products like yoghourts or fermented dairy beverages, chewing-gums, candies , petfoods and oral supplements.

12. Use of the inhibitors according to claims 7 and 8 or inhibitory agent according to claim 1 for the manufacture of a drug for reducing feline osteoclastic resorptive lesions.

13. Pharmaceutical composition comprising an effective amount of inhibitors according to claims 7 and 8 or inhibitory agent according to claim 1 for reducing activities and/or functions of metalloproteinases, in particular matrix-metalloproteinases.

A

B

FIGURE 1

SEQUENCE LISTING

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